

Major structural differences between pokeweed antiviral protein and ricin A-chain do not account for their differing ribosome specificity

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Pokeweed antiviral protein (PAP) and the A-chain of ricin (RTA) are two members of a family of ribosome-inactivating proteins (RIPs) that are characterised by their ability to catalytically depurinate eukaryotic ribosomes, a modification that makes the ribosomes incapable of protein synthesis. In contrast to RTA, PAP can also inactivate prokaryotic ribosomes. In order to investigate the reason for this differing ribosome specificity, a series of PAP/RTA hybrid proteins was prepared to test for their ability to depurinate prokaryotic and eukaryotic ribosomes. Information from the X-ray structures of RTA and PAP was used to design gross polypeptide switches and specific peptide insertions. Initial gross polypeptide swaps created hybrids that had altered ribosome inactivation properties. Preliminary results suggest that the carboxy-terminus of the RIPs (PAP 219–262) does not contribute to ribosome recognition, whereas polypeptide swaps in the amino-terminal half of the proteins did affect ribosome inactivation. Structural examination identified three loop regions that were different in both structure and composition within the amino-terminal region. Directed substitution of RTA sequences into PAP at these sites, however, had little effect on the ribosome inactivation characteristics of the mutant PAPs, suggesting that the loops were not crucial for prokaryotic ribosome recognition. On the basis of these results we have identified regions of RIP primary sequence that may be important in ribosome recognition. The implications of this work are discussed.

Keywords: ribosome-inactivating proteins; pokeweed antiviral protein; ricin; ribosome recognition; *N*-glycosidase.

Many plants, fungi and bacteria produce ribosome-inactivating proteins (RIPs) which can attack and catalytically inactivate eukaryotic ribosomes and thereby inhibit protein synthesis. The physiological role of these proteins is unknown although it is widely believed that plant RIPs play roles in defence, e.g. as potential antiviral or antifungal agents (Lord et al., 1991). RIPs are characterised by their ability to remove an invariant adenine base from a conserved loop in 28S rRNA (Endo and Tsurugi, 1987). This loop is involved in binding elongation factors and its depurination leads to irreversible inactivation of the 60S ribosomal subunit and the cessation of protein synthesis.

Classically, RIPs have been categorised into two families based on their structural characteristics. Pokeweed antiviral protein (PAP) from *Phytolacca americana* is a representative of the type 1 family of RIPs, all of which are single chain *N*-glycosidases with molecular mass around 30 kDa. In addition to the type 1 class of RIPs, some plants produce heterodimeric proteins termed type 2 RIPs. These have an A chain that appears to be structurally and functionally related to the type 1 RIPs, disulfide

linked to a sugar-binding B chain. The majority of the type 2 RIPs, as exemplified by the castor oil seed toxin ricin, are potent cytotoxins owing to the cell binding ability of the B chain which promotes the obligatory first step in toxin uptake. The type 1 RIPs, in contrast, are not cytotoxic since they lack a means of initially binding to the surface of cells. If introduced into cells by an alternative carrier then cytotoxicity is observed. Unusually, two type 2 RIPs have been shown to exhibit extremely poor cytotoxicity but *in vitro* protein synthesis inhibition is equivalent to other type 2 RIPs (Girbes et al., 1993a,b).

A surprising finding in recent years has been that several type 1 RIPs, including PAP, show activity towards not only eukaryotic ribosomes but also prokaryotic ribosomes (Hartley et al., 1991). To date, no type 2 RIPs have been shown to inactivate prokaryotic ribosomes. Depurination of *Escherichia coli* 23S rRNA occurs at A2660, in a functionally equivalent position to the target adenine of eukaryotic 26/28S rRNA (A4324 in rat liver). The location of the target adenine within the rRNA structure is equivalent in both eukaryotic and prokaryotic ribosomes and was shown by Endo et al. (1987) to lie in a highly conserved 14-base purine-rich sequence (α -sarcin loop). Studies of the kinetics of RTA-catalysed depurination have determined the K_m and k_{cat} for eukaryotic ribosomes to be approximately 1 μ M and 1500 min⁻¹ respectively (Endo and Tsurugi, 1987). Although RTA is inactive towards intact prokaryotic ribosomes, depurination of naked 23S rRNA by RTA has been described (Endo and Tsurugi, 1987). Since the prokaryotic rRNA can serve as a sub-

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Abbreviations. ID₅₀, concentration of protein for 50% depurination; PAP, pokeweed antiviral protein; RTA, ricin A-chain; RIP, ribosome inactivating protein.

Enzyme. *N*-glycosidase (EC 3.5.1.52).

strate for RTA-dependent N-glycosidase activity when stripped of ribosomal proteins, and the rRNA target sequence is conserved, the molecular basis of this difference in RIP specificity is intriguing.

Studies have also shown that the three-dimensional structural alignments of PAP and ricin A chain are very similar and the organisation of the putative active-site region is highly conserved. However, a small number of polypeptide regions were identified as having sufficiently different tertiary structure to warrant investigation as possible ribosome-specificity determinants (Monzingo et al., 1993). It has been suggested that regions of RIP protein structure, possibly quite distinct from the active site, may determine ribosome specificity. The aim of this present study is to investigate this possibility by using RTA and PAP as model proteins for RIPs that are only active against eukaryotic ribosomes (RTA) or have activity against both eukaryotic and prokaryotic ribosomes (PAP). Gross polypeptide swaps and specific peptide swaps have been generated to create RTA/PAP hybrid proteins for examination of their ability to inactivate ribosomes.

MATERIALS AND METHODS

Construction of polypeptide hybrids. Hybrids were constructed using the PAP template described previously (Chaddock et al., 1994) which has a TGA codon inserted after the codon for Thr262 and a deletion of the sequence coding for the 29-amino-acid C-terminal extension. Nucleotide and amino acid numbering are derived from the previously reported PAP cDNA (Lin et al., 1991) and ricin cDNA (Lamb et al., 1985) sequences, with numbering initiated at the first codon of the mature protein sequence. Site-specific mutagenesis was performed using the T7-GEN *in vitro* mutagenesis system.

In order to construct templates for the polypeptide swaps, M13 clones were prepared by ligation of a pET *XbaI*–*Bam*HI fragment from pETPAPSTOP and pETRTA into M13mp18. Clones were initially created in M13, sequenced and the *XbaI*–*Bam*HI fragment isolated for ligation into similarly cut pET11d. Swap 1 clones (i.e. the N-terminal portions) were constructed from PAP and RTA templates mutated to create a *NheI* site at base 192 (PAP). Mutant M13 was cleaved with *NheI*/*Hind*III and the small DNA fragments swapped. In addition to the desired mutation, this strategy resulted in the mutation Met65 → Ala in PAP. Swap 2 clones (i.e. central region) were created from templates having *NheI* and *Csp*45 sites inserted at base positions 192 and 379, respectively. The respective *NheI*–*Csp*45 DNA fragments were swapped. This strategy resulted in the mutation Leu126 → Phe in both swap 2 hybrids. Swap 3 clones (i.e. C-terminal region) were created using templates mutated to introduce *Csp*45 sites at base 654, resulting in secondary mutants Ala218 → Ser (PAP) and Glu220 → Asp (RTA). *Hind*III–*Csp*45 small DNA fragments from these mutated templates were swapped to construct the swap 3 series.

Construction of peptide swap mutants. Three peptide swaps (PAP80, PAP110 and PAP122) were constructed using the following procedures. pET80 was created by insertion of a double-stranded oligonucleotide linker into a PAP template which had been mutagenised to insert two restriction sites. Two mutated M13PAPSTOP templates with a *NruI* site at base position 232 and a *KpnI* site at 260 (M13JAC1 and M13JAC2, respectively) were prepared. A linker oligonucleotide (created by hybridisation of GCTGGAAATTCGTAC with GAATTC-CAGC) was ligated to a 661-bp M13JAC2 *KpnI*–*Bam*HI fragment. The hybrid was ligated to a 6672-bp M13JAC1 *Bam*HI–*NruI* fragment, transformed into *E. coli* TG2 and sequenced.

Clone 110 was constructed using a PCR method. A 485-bp PAP sequence, amplified using oligonucleotide TTAAGTGATGTT-CAAAATAGTAAAAACAT and JACEND (Chaddock et al., 1994), was digested with *Bam*HI and ligated to *Bam*HI/*Afl*III-cut M13JAC5 (containing an *Afl*III site mutated into the PAP sequence at base 315). The PCR fragment/M13 hybrid was treated with mungbean nuclease to blunt the *Afl*III site, then ligated to form M13110. M13122 was constructed by mutagenesis of M13PAPSTOP with the T7-GEN system and the mutagenic oligonucleotide AACATAAACTTTGGTGGTAATTATGATAGATTGGAATCAAAAG. All M13 clones were sequenced fully before mutant DNA was cloned into pET11d by digestion of the M13 clone with *XbaI*/*Bam*HI.

Northern blotting. Northern Blotting of rRNA onto Hybond-N membrane was performed essentially as described by Sambrook et al. (1989) using Pharmacia VacuGene XL apparatus. Hybridisation of ³²P-end-labelled oligonucleotide probes (75 pmol, approximately 2 × 10⁶ dpm) specific to the 3' end of the 28S and 23S rRNA was performed overnight in fresh hybridisation solution (0.015 M NaCl, 0.0015 M sodium citrate, pH 7, 0.3 % SDS) at 37°C/42°C respectively. Filters were washed twice for 30 min with 0.03 M NaCl, 0.003 M sodium citrate, 0.1 % SDS prior to exposure to X-ray film at –70°C.

Miscellaneous methods. All clones were transformed into *E. coli* BL21(DE3)pLysS and were maintained as glycerol stocks at –70°C. Protein expression and prokaryotic rRNA extraction are described elsewhere (Chaddock et al., 1994). Protein purification, N-glycosidase assay, and *in vitro* transcription/translation techniques were essentially as previously described (Chaddock and Roberts, 1993). Other standard laboratory methods were as in Sambrook et al. (1989).

RESULTS

Polypeptide switches. Hybrids were constructed with the potential to encode large regions switched in polypeptide content to investigate their contribution to ribosome specificity. The validity of such an approach was confirmed with the later observations from X-ray structure analysis of PAP and RTA demonstrating the highly conserved tertiary structure. These gross changes were made by introduction of specific restriction enzyme sites into the PAP and RTA DNA sequences, digestion of mutant DNA, and cloning DNA fragments into the respective partner. Restriction sites were chosen to minimise codon mutations. Sequences between PAP residues 1–63, 64–126, and 219–262 were exchanged with RTA equivalents 1–71, 72–126, and 222–267 to investigate the contribution to ribosome recognition of these gross changes.

The first swap representing amino acids 1–63 was designed to investigate the N-terminal region, which is relatively low in conservation between PAP and RTA and has previously been implicated in having potential ribosome-interactive properties (Watanabe and Funatsu, 1986; Mlsna et al., 1993). The second region (64–126) was changed to investigate several phenomena. These include the effects of altering a substantial amount of the β -sheet structure on one side of the active site but not affecting the key catalytic residues, and investigating the importance of the putative RNA recognition motif present in residues 78–84 in RTA and 70–76 in PAP identified by primary sequence similarity in a maize RIP by Bass et al. (1992). In addition, swap 2 contained the peptide regions of loops 80 and 110 that were later individually mutated (see below). The third swap assessed the contribution of the C-terminal region of the protein, leaving all the major catalytic residues untouched. The C-termini of PAP and RTA constitute a region of low secondary structure and are

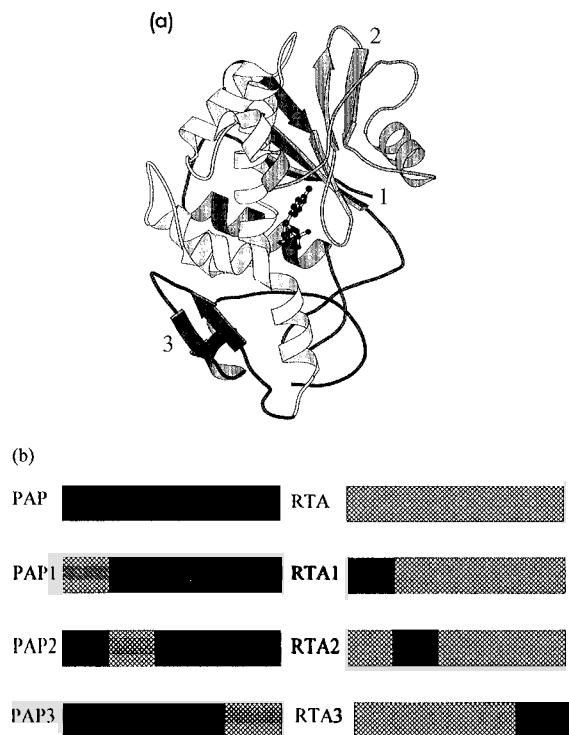


Fig. 1. Polypeptide swap structure. Three PAP mutants, PAP1 (1–63), PAP2 (64–126) and PAP3 (219–262) were created by substitution of the PAP residues indicated by the equivalent RTA residues. (a) Location of polypeptide swaps in PAP tertiary structure. The swapped regions in PAP1, PAP2 and PAP3 are highlighted in dark grey, light grey and black respectively. (b) Schematic representation of swap constructs. PAP-derived polypeptide is highlighted in black and RTA-derived polypeptide in hatching.

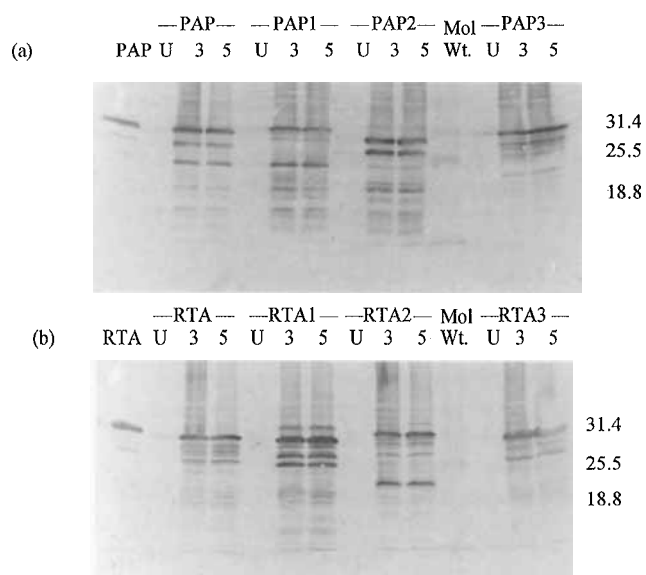


Fig. 2. Expression of polypeptide swap mutants in *E. coli* and analysis by Western blotting. Samples of expression culture were removed prior to induction (U), 3 h post-induction (3) and 5 h post-induction (5) and analysed by SDS/PAGE and Western blotting with anti-PAP antibodies (a) or anti-RTA antibodies (b). In both cases 100 ng recombinant wild-type protein was blotted as control. Approximate molecular masses (in kDa) are indicated on the right.

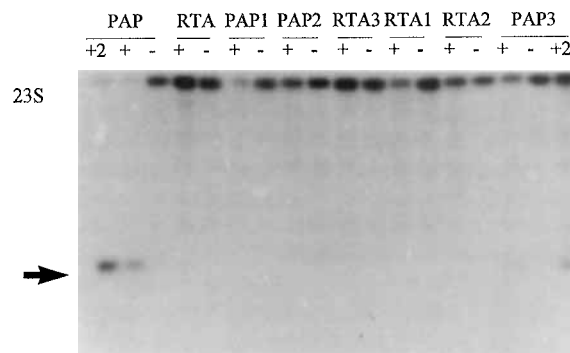


Fig. 3. N-glycosidase activity of PAP and RTA mutants during *E. coli* expression. rRNA from expression-culture ribosomes was isolated and 4 µg was treated (+) or not treated (–) with aniline. Samples were electrophoresed on agarose/formamide gels prior to Northern blotting onto nylon membrane. The 3' fragment of the 23S rRNA was identified by hybridisation to a specific oligonucleotide probe. Fragments of 23S rRNA released by aniline cleavage of depurinated RNA are indicated by an arrow. Increased amounts of rRNA were loaded in lanes identified as +2 for ease of visualisation.

poorly conserved. This may reflect the fact that RTA has a requirement to bind to ricin B-chain, whereas the type 1 RIP PAP does not. Alternatively, the C-terminal region may have the characteristics to interact with prokaryotic ribosomes which have been lost by the type 2 RIPs during evolution of the A-chain/B-chain interface. Fig. 1a indicates the location of the swap regions in the tertiary structure of PAP and Fig. 1b shows a schematic of the primary structure. Nomenclature of these hybrids is based on the name of the majority protein content of the hybrid followed by the domain swap number. For example, PAP with the amino-terminal peptide from RTA would be termed PAP1, while that contains the C-terminal peptide from RTA would be PAP3; PAP2 would be the 'central' swap.

Hybrids were constructed in M13mp18 for convenient sequencing and the subcloned into pET11d for *in vitro* and *in vivo* expression. This expression system is tightly regulated and has previously been used to express highly toxic proteins with success (Studier and Moffatt, 1986). *In-vitro*-generated transcripts were translated in a wheat germ cell-free system and were shown to give products of the expected molecular mass (data not shown). The DNAs were transformed into BL21(DE3)pLysS for expression experiments, and the expression of full-length hybrids was assessed by Western blotting of crude *E. coli* lysates using anti-PAP and anti-RTA sera. Fig. 2 shows that, in all cases, protein was expressed following isopropyl β -D-thiogalactopyranoside induction, though there was a certain amount of proteolytic activity that led to some specific degradation of hybrids. Mutants were expressed to similar levels and there was no observable difference in the culture growth rates (data not shown).

The ability of the hybrids to inactivate prokaryotic and eukaryotic ribosomes was investigated. rRNA was isolated from bacterial ribosomes extracted from the hybrid expression system both before and after a 3-h induction with isopropyl β -D-thiogalactopyranoside. This rRNA was treated with aniline and depurination assessed. Fig. 3 shows a Northern blot of such an experiment. Northern blotting and radioactive probing was necessary to visualise and confirm depurination. Protein was expressed to equivalent levels from all the constructs as ascertained by Western blot analysis of culture after 3-h induction. Depurination of host ribosomes was observed with PAP and PAP3 but not in the cases of PAP1, PAP2, RTA1, RTA2 or RTA3. This suggests that PAP1 and PAP2, both predominantly PAP-like, had either reduced or no ability to inactivate prokaryotic ribosomes.

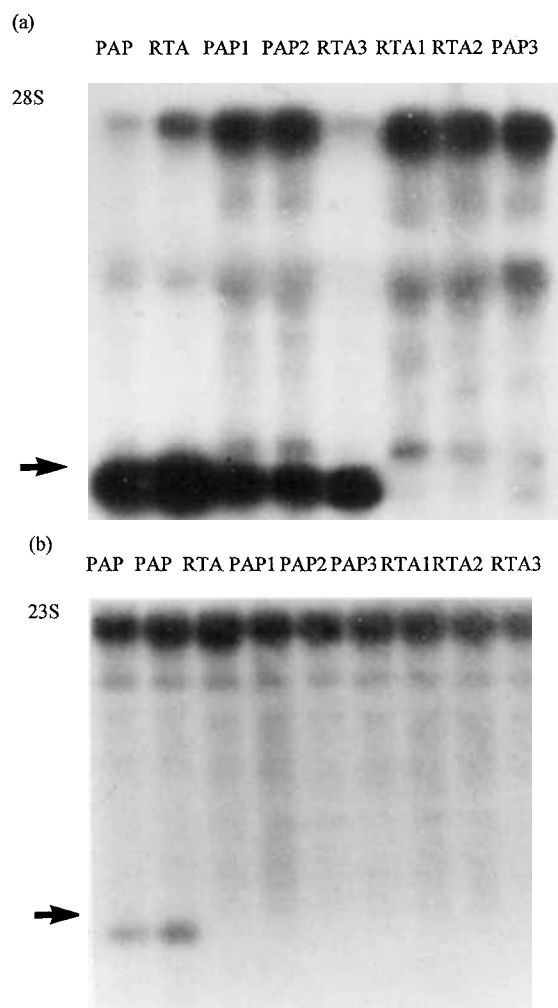


Fig. 4. N-glycosidase activity of PAP and RTA mutants toward eukaryotic and prokaryotic ribosomes in a non-translating *in vitro* system. Soluble protein was prepared from *E. coli* sonicates and equivalent amounts assessed for depurination ability. Northern blots of aniline-treated rRNA were probed with specific probes for the 3' termini of the eukaryotic 28S rRNA (a) and prokaryotic 23S rRNA (b). The RIP-specific RNA fragment released following aniline cleavage is indicated by an arrow.

In order to test if the proteins expressed in this system retained N-glycosidase activity toward isolated eukaryotic ribosomes, crude sonicates were prepared after induction of expression for 3 h. Subsequent $100\,000\times g$ centrifugation prepared a soluble fraction of each hybrid which was used to test for the ability to inactivate reticulocyte ribosomes *in vitro*. Equivalent amounts of soluble protein from each mutant was analysed. As indicated in Fig. 4, only constructs PAP1, PAP2, RTA3, RTA and PAP showed activity in this assay. Northern blot analysis was performed to increase the sensitivity of RNA visualisation, and this analysis confirmed that only the hybrids identified above were active. The data for PAP3 were not consistent with its observed activity towards prokaryotic ribosomes during expression. When proteins including PAP3 were extracted from *E. coli* and added to isolated prokaryotic ribosomes *in vitro*, only wild-type PAP depurinated rRNA (Fig. 4). A summary of these domain swap results is shown in Table 1.

Peptide swaps. Comparison of the tertiary structures of PAP (Monzingo et al., 1993) and RTA (Katzin et al., 1991) led to the identification of three peptide regions that were noticeably

dissimilar between RTA and PAP. They were located within the polypeptide swaps that had altered properties and therefore were potentially important. These regions were named 80, 110 and 122 to describe the approximate amino acid positions in PAP. Loops 80 (Asp78–Arg86) and 110 (Cys106–Val113) are located in the regions between β -strands d and e and between α -helix B and β -strand f, respectively, whereas loop 122 (residues Asp120–Thr125) forms a 'lid' structure at the entrance of the active site, displaying a different structure and charge distribution between PAP and RTA. To investigate if activity towards prokaryotic ribosomes could be reduced, as had been seen with PAP1 and PAP2, the respective RTA sequences were inserted into the PAP backbone to create mutant PAP proteins. The positions of the peptide swaps in the tertiary structure are indicated in Fig. 5 and the amino acid changes are shown in the accompanying legend.

Conversion of the PAP-like motifs to the equivalent RTA-like motifs was performed by DNA manipulation as described and constructs were prepared in the *E. coli* expression vector pET11d. All three mutants were shown to express equivalent quantities of protein after induction with isopropyl β -D-thiogalactopyranoside (Fig. 6a). No significant differences in growth characteristics were observed following induction of toxic and non-toxic proteins, therefore monitoring the absorbance during expression did not provide a good indicator of relative activities of the hybrids. An improved indication of activity was provided by *E. coli* cell viability, as measured by a plating assay. Viability was reduced to less than 0.1 % of the pre-induction levels after 3 h of PAP expression (data not shown). Ribosomal RNA from the expression cultures of the three mutants was isolated and checked for depurination. In all cases the ribosomes had been depurinated indicating that the protein was active to the host ribosomes during expression (data not shown). In order to assess activity more accurately, the mutant proteins were purified by cation-exchange chromatography. PAP80 (Fig. 6b) and PAP122 were successfully purified to homogeneity as assessed by silver staining samples following SDS/PAGE. PAP110 was not obtained fully pure using similar techniques but was highly enriched. The concentration of PAP110 within the semi-purified sample could be estimated from densitometry of stained SDS/polyacrylamide gels, as was done also for the fully purified mutant proteins PAP80 and PAP122.

Mutant proteins were assayed for activity toward isolated rabbit reticulocyte ribosomes and isolated *E. coli* ribosomes *in vitro*. *E. coli* ribosomes have previously been shown to be approximately 100–500-fold less sensitive to RIPs than eukaryotic ribosomes (Hartley et al., 1991). Assays were performed in Endo buffer in the absence of additional factors. Appropriate concentrations of toxin were incubated with $1\ \mu\text{g}/\mu\text{l}$ ribosomes for 30 min at 30°C , rRNA was extracted and the amount of depurination estimated by densitometry of ethidium-bromide-stained gels (Chaddock and Roberts, 1993). Since it was not the intention of this analysis to prepare kinetic parameters for each mutant, but rather to investigate their relative activities toward the two ribosome types, these assay conditions were satisfactory. Fig. 7 shows a titration of purified recombinant PAP versus PAP80 and clearly demonstrates that the mutant PAP80 protein does not have reduced activity towards prokaryotic ribosomes. For each mutant toxin a similar titration was performed and the amounts of depurination assessed. Comparison of the activities of the toxins was made possible by estimating the ID_{50} (concentration of toxin for 50 % depurination) from graphical analysis of the amount of depurination resulting from various toxin concentrations. For each measurement of hybrid activity, a control experiment was performed using purified wild-type PAP and an ID_{50} calculated. The mutant ID_{50} was then compared to the wild-

Table 1. Summary of activity assessments of polypeptide swaps.

Ribosomal substrate	Depurination activity of protein							
	RTA	PAP	PAP1	PAP2	PAP3	RTA1	RTA2	RTA3
Eukaryotic <i>in vitro</i> (non-translating)	yes	yes	yes	yes	no	no	no	yes
Prokaryotic <i>in vitro</i> (non-translating)	no	yes	no	no	no	no	no	no
Prokaryotic host (translating)	no	yes	no	no	yes	no	no	no

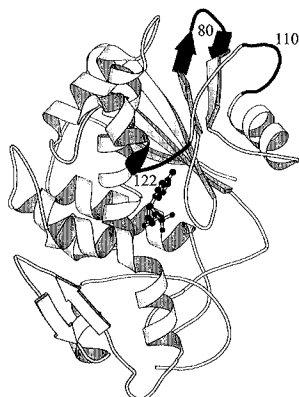


Fig. 5. The location of peptide loop swaps in the tertiary structure of PAP. Mutant PAP proteins were created by replacement of peptide regions by the RTA equivalent sequences. Proteins were termed PAP80, PAP110 and PAP122 for D⁷⁸PFETNKCR⁸⁰ to AGNS, C¹⁰⁶PNANSRV¹¹³ to TDVQN and D¹²⁰SRYPY¹²⁵ to GGNVDR respectively. The location of the proposed active site at the centre of the figure is shown by the position of the formycin 5'-monophosphate ring structure.

type and the N-glycosidase activity of the mutants calculated as a percentage of wild-type activity (Table 2).

From the analysis it is apparent that RTA sequences inserted into PAP had not significantly affected the ability of the mutant proteins to inactivate prokaryotic or eukaryotic ribosomes. In all three cases, the activity of the mutant was within one order of magnitude of wild-type PAP during these assays. Since RTA is not active towards *E. coli* ribosomes at concentrations up to 10000-fold greater than concentrations needed to inactivate reticulocyte ribosomes (Ready et al., 1991), we can assume that the mutant PAP hybrids are not greatly affected in prokaryotic ribosome depurination. Clearly, the swapped peptide regions alone do not account for the prokaryotic ribosome specificity of PAP.

DISCUSSION

Although the key catalytic residues present in the active site of RIPs are always conserved, and the target adenine residue they remove from rRNA is present in an absolutely conserved base sequence, the target ribosome specificity for different RIPs can vary dramatically. The work presented here represents an initial attempt to determine whether the structural features of RIPs govern their ribosome specificity. It was decided to use RTA and PAP to investigate their documented differences in inactivation of bacterial (prokaryotic) and rabbit reticulocyte (eukaryotic) ribosomes. The availability of cDNA clones and the later information derived from the tertiary structures of PAP and RTA were deciding factors in the choice of RIPs. Many workers have described the specificity of RIPs in relation to their ability to inactivate self and non-self ribosomes, and have attempted to

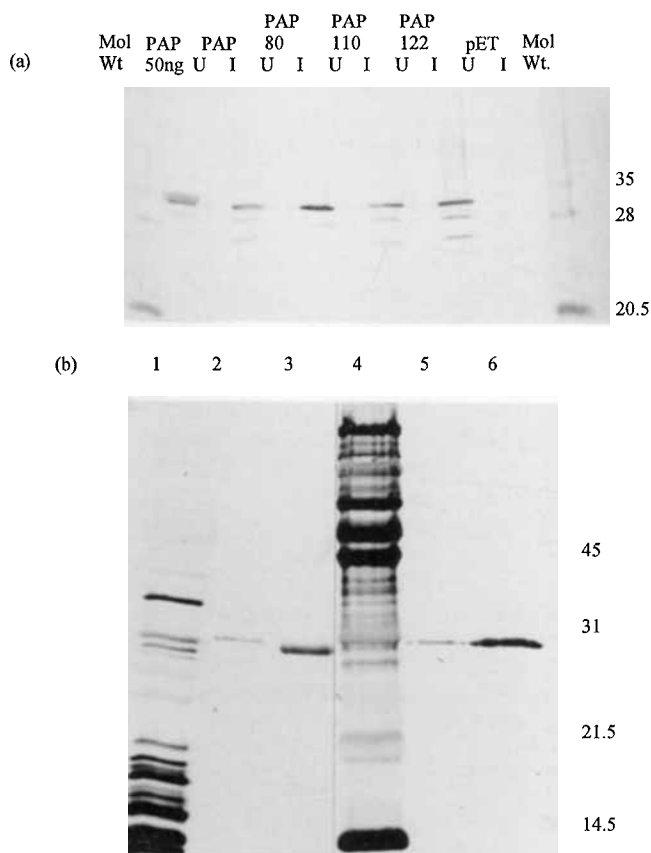


Fig. 6. Expression and purification of PAP peptide loop mutants. (a) Analysis of PAP peptide loop mutants expressed in BL21(DE3)pLysS by SDS/PAGE and Western blotting with anti-PAP antibodies. 1-ml samples of culture were removed prior to (U) and 3 h post-induction (I) with isopropyl- β -D-thiogalactopyranoside, centrifuged at 13000 \times g for 5 min, and samples prepared for electrophoresis. Samples were also taken from *E. coli* transformed with the expression vector alone (pET). (b) Purification of PAP80 by cation-exchange chromatography. Samples of *E. coli* culture (lane 1), 100 ng PAP protein from *Phytolacca americana* (lanes 2 and 5), purified recombinant PAP80 (lane 3) and purified recombinant PAP (lane 6) were visualised by silver staining following SDS/PAGE. Molecular mass markers are shown in lane 4 and approximate molecular masses (in kDa) are indicated on the right.

explain why RIPs have evolved a specificity for ribosomes (Prestle et al., 1992; Taylor et al., 1994; Wong et al., 1995). However, little work has been described where the features necessary for ribosome recognition and interaction have been investigated. Rather, most mutagenesis experiments have concentrated on the determination of the catalytic mechanism (Kim and Robertus, 1992; Chaddock and Roberts, 1993). A recent study by Morris and Wool (1994) described the effects of deletions on helix D in RTA and concluded that none of the residues in this region (Asn141–Tyr152) were involved in ribosome recogni-

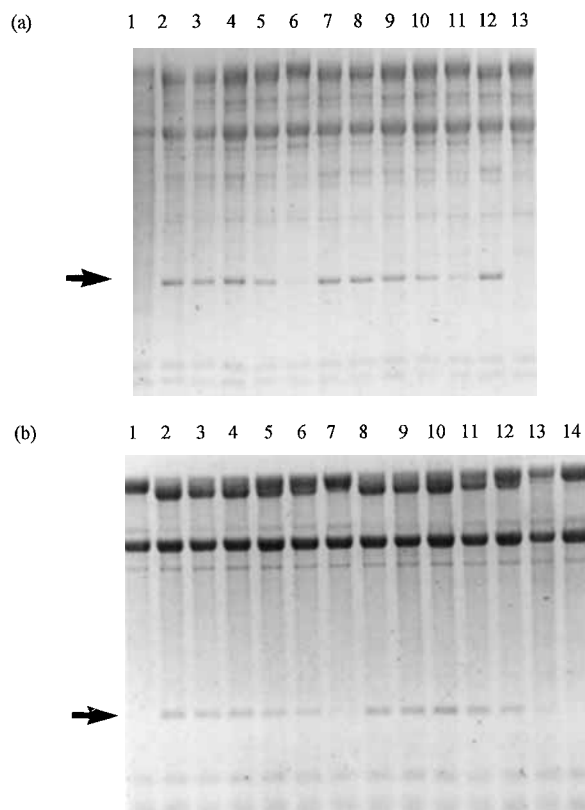


Fig. 7. Assessment of the N-glycosidase activity of purified PAP and PAP80 towards eukaryotic and prokaryotic ribosomes. (a) 30 μg isolated rabbit reticulocyte ribosomes were treated with PAP (lanes 2–6) and PAP80 (lanes 7–11) for 30 min at 30°C, RNA isolated and the aniline-treated RNA visualised following electrophoresis by ethidium bromide staining of agarose/formamide gels. Final toxin concentrations of 10 ng/μl (lanes 2 and 7), 5 ng/μl (lanes 3 and 8), 1 ng/μl (lanes 4 and 9), 0.1 ng/μl (lanes 5 and 10) and 0.01 ng/μl (lanes 6 and 11) were used. Lanes 1 and 13 show non-aniline-treated samples of 10 ng/μl PAP and 10 ng/μl PAP80 respectively. Lane 12 shows depurination resulting from incubation of 3.3 ng/μl RTA. (b) 30 μg isolated *E. coli* ribosomes were treated with PAP (lanes 2–7) and PAP80 (lanes 8–13) as above. Final toxin concentrations of 10 ng/μl (lanes 2 and 8), 6.7 ng/μl (lanes 3 and 9), 5 ng/μl (lanes 4 and 10), 3.3 ng/μl (lanes 5 and 11), 1.7 ng/μl (lanes 6 and 12) and 0.8 ng/μl (lanes 7 and 13) were used. Lanes 1 and 14 show non-aniline-treated samples of 10 ng/μl PAP and 10 ng/μl PAP80, respectively. The rRNA fragment released is indicated by an arrow.

Table 2. N-glycosidase activity of purified PAP80, enriched PAP110 and purified PAP122 relative to wild-type PAP.

Ribosome substrate	N-glycosidase activity of		
	PAP80	PAP110	PAP122
	% wild-type PAP		
Eukaryotic (non-translating)	100	14	26
Prokaryotic (non-translating)	100	30	13

tion or catalysis. Habuka et al. (1992), investigating the properties of active-site mutants of *Mirabilis* antiviral protein, showed that certain mutants do have a reduced ability to inactivate prokaryotic ribosomes. In addition, it was recently suggested that

the electrostatic potential of the residues surrounding the active site was important in determining ribosome interaction (Ago et al., 1994). However the study did not go further to investigate whether this charge distribution determined ribosome specificity.

We (Katzin et al., 1991; Monzingo et al., 1993), and others (Husain et al., 1994; Weston et al., 1994), have taken the view that the recognition of substrate may involve residues distant from the active site in regions of the protein that may interact with ribosomal proteins to determine specificity. There are several lines of evidence to support this hypothesis. First, the key active-site residues of all RIPs studied are conserved, as are their positions in the active site. Second, the ribosomal RNA sequences are highly conserved in the target area leading to little differences in the RNA substrate. Third, *E. coli* rRNA is depurinated by RTA, albeit poorly, after removal of ribosomal proteins (Endo and Tsurugi, 1987) suggesting that, in the absence of ribosomal-proteins, prokaryotic rRNA does indeed adopt a conformation, possibly RTA-induced, suitable for depurination. Since RTA possesses the correct active-site structure to depurinate *E. coli* rRNA, we hypothesise that the deciding factor for depurination *in vivo* is the presence of ribosomal proteins and their relative ability to interact with RIPs. It appears that certain RIPs have fortuitously evolved a surface compatible for the interaction with prokaryotic ribosomes.

In order to address the question of ribosomal specificity, a series of polypeptide and peptide swaps between PAP and RTA were constructed. A random mutagenesis approach was not adopted here since we rationalised that single-residue changes brought about by a random approach may not be sufficient to determine substrate recognition. Further, such an approach would generate mostly structural and active site mutants which would be laborious to distinguish from mutants of interest. Rather, it seemed more likely that a patch of residues may create a suitable recognition determinant. Polypeptide swap hybrids were prepared in order to transfer larger patches of potential surface-interactive zones to test this hypothesis.

The results obtained for the polypeptide changes were interesting. Of the polypeptide switch mutants, only PAP3 was active towards prokaryotic ribosomes during expression in *E. coli*. However, no activity against ribosomes from *E. coli* or rabbit reticulocyte was observed following isolation of PAP3 from the expression culture and *in vitro* activity assessment. This implies that PAP3-dependent depurination during expression may be a transient activity that could only be observed briefly after translation before aberrant folding and loss of activity. It was shown that, although soluble PAP3 could be recovered, the hybrid was very sensitive to proteinase K digestion (in contrast to PAP and RTA) suggesting poor/alttered folding properties (data not shown). The complementary hybrid RTA3 was active toward eukaryotic ribosomes but was not active to prokaryotic ribosomes as one would expect of a hybrid protein that was predominantly RTA. The results from RTA3 and PAP3 suggest that the C-terminus of PAP does not contain crucial prokaryotic ribosome recognition determinants.

In contrast to PAP3, PAP1 and PAP2 did not inactivate prokaryotic ribosomes, either during translation in *E. coli* or in an *in vitro* assay. However, they exhibited substantial eukaryotic ribosome inactivation properties *in vitro*, suggesting that the active-site co-ordination was intact. Therefore, it appears that these mutants have been altered in prokaryotic ribosome recognition, suggesting that interactive zones lie within the first 126 residues of the protein. Since these swapped regions are non-overlapping, the possibility exists that each region, when mutated separately, affects just a component of the recognition zones. It may be that only when both components are intact can prokaryotic ribosomes be depurinated. Alternatively, it is pos-

sible that the conversion of one region has had a deleterious effect on crucial recognition residues present in the second, such that the observed effect is the same.

RTA1 and RTA2, complementary swaps to PAP1 and PAP2, were inactive in both the eukaryotic and prokaryotic assay systems tested, suggesting that, although soluble protein could be prepared, these proteins were non-functional. PAP1 and PAP2 were active against reticulocyte ribosomes, suggesting that the PAP backbone may have an inherently greater ability to accept changes in its structure compared to RTA. Deletion mutagenesis performed previously by Morris and Wool (1992) suggested that RTA has the ability to accept primary sequence perturbation in many positions without affecting *in vitro* N-glycosidase activity during translation. Insertion of large polypeptide regions will impose a different constraint to deletions, and it may be that PAP is better equipped to accommodate these replacements.

In order to examine further the results obtained for PAP1 and PAP2, a series of peptide swaps were created using information from the X-ray structures of PAP and RTA. Analysis of the tertiary structures revealed striking differences between the PAP and RTA tertiary structures within the potentially important N-terminal region. Loops 80 and 110 are noticeably less pronounced in RTA and do not project to solvent as clearly in the case of PAP. Under suitable conditions these loop structures in PAP are disulfide-bonded together, though this is not essential for activity. Examination of the modelled three-dimensional structure of *Mirabilis* antiviral protein (from *Mirabilis jalapa*), a protein also shown to be active against bacterial ribosomes, had amino acid extensions in these areas, suggesting that they could be important in prokaryotic recognition. These regions were therefore strong candidates for ribosome specificity determinants. The proteins were otherwise highly conserved at the level of the α -carbon backbone. However, directed switching of specific peptide loop regions in PAP to the equivalent residues in RTA did not abolish ribosome inactivation (Table 2). Therefore, it must be concluded that these regions are not involved in the determination of prokaryotic ribosomes specificity and that other features of the swapped polypeptides in PAP1 and PAP2 are responsible. The reason for the existence of these polypeptide loop extensions is therefore unclear.

Examination of the X-ray structure in the light of these peptide and polypeptide swap experiments has revealed two peptide regions of interest that correlate with the ability of the hybrids to inactivate prokaryotic ribosomes. Regions 48–55 and 95–101 in PAP are surface-located towards the outer face of the active-site cleft and are both present in hybrids that are active toward prokaryotes. If either or both are replaced by the equivalent RTA sequence then prokaryotic depurination is lost, hence PAP1, PAP2, RTA1, RTA2 and RTA3 were not active, whereas PAP3, PAP80, PAP110 and PAP122 are active. In this region, the α -carbon positions in PAP and the equivalent atoms in RTA are observed as different surface loop structures, with the RTA loops being slightly more extended than PAP. This altered backbone structure affects the organisation and orientation of the side chains. In the case of RTA, the side chain of Arg48 could form an ion pair with the side chain of Glu99. The acidic side chains of Asp75, Asp100 and Glu102 probably remain unpaired in this region. With PAP, the side chain of Lys48 may ion pair with Asp100; and Glu97 may form an ion pair with Arg67, whereas Asp92 probably remains unpaired. Comparison of the electrostatic surfaces of PAP and RTA using GRASP (Nicholls et al., 1991) indicated that the charge organisation on the surface of these RIPs is different, particularly in the 48–55 and 95–101 area. Examination of the electrostatic potential of X-PLOR energy minimised models (Brunger, 1988) of the various PAP-RTA hybrids revealed that only PAP3, which was active toward pro-

karyotic ribosomes, has a virtually identical charge pattern to PAP in this area. It is unclear at present whether the backbone structure and subsequent side-chain organisation or the charge differences alone are important. Further experimental work will be required to investigate if these regions are involved in recognition and to decide whether the charge characteristic is the major factor.

This work represents an initial attempt to define regions of RIP structure that may determine ribosome specificity. Future work will focus on the specific areas speculated above to investigate any contribution to ribosome recognition. The increasing number of reported crystal structures for RIPs will greatly assist this exercise since more accurate comparisons can then be made. Additional activity data for RIP activity will prove to be invaluable in studies of this type. In the longer term, it is hoped that a greater understanding of RIP-ribosome recognition will allow the creation of hybrid RIPs with defined specificities for use in the fields of therapeutics, plant-pathogen defence and ribosomal structural studies.

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